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RESEARCH REPORT

Inheritance of heat stable esterase in near isogenic lines and functional classification of esterase in silkworm *Bombyx mori*

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Abstract

Esterases are ubiquitous in living organisms and perform multiple functions in animals, plants, insects and microorganisms. Insect esterases broadly perform physiological and defense functions. The present work aims towards identifying heat stable esterase in the hemolymph of near isogenic lines (NILs) and their parents, and further to classify esterases based on substrate-inhibition reactions in silkworm, *Bombyx mori.* Five different α-esterases *viz*. Est-1, Est-2, Est-3, Est-4 and Est-5 were observed in this study. Of them, Est-1 and Est-2 were monomorphic and, Est-3, Est-4 and Est-5 were polymorphic. Heat stability studies revealed Est-2 and Est-3 as heat stable and, Est-1, Est-4 and Est-5 as heat liable. Through inhibition analysis, Est-1 was identified as cholinesterase and Est-5 as carboxylesterase because their activity was totally inhibited, respectively, by eserine sulphate and phenylmethylsulfonyl fluoride (PMSF) while Est-3 was inhibited by both the inhibitors. The Est-2 and Est-4 were unaffected by both PMSF and eserine sulphate. The isozyme patterns of breed specific as well as heat stable esterases supports the variations in the survival percentage of silkworm breeds at high temperatures. This study enhances the knowledge on esterase-mediated thermotolerance in *B. mori*.

Key Words: *Bombyx mori*; hemolymph; carboxylesterase; cholinesterase; thermotolerance

Introduction

Esterases (EC 3.1.1.X) are ubiquitously present in all living organisms. They form a group of hydrolases that perform multiple functions in plants, animals, insects and microbes. In particular, insect esterases are involved in digestion of nutritional material and mediation of insecticide resistance (Oakeshott *et al*., 1993; Shiotsuki and Kato, 1999; Amanullah *et al*., 2010). Furthermore, they also participate in degradation of pheromone and hydrolysis of the juvenile hormone (JH) at different life-stages of insects (Taylor and Radic, 1994).

The specificity between esterase and substrate varies in insects, therefore insect esterases are classified based on their reactions with different substrates as α- or β-esterases (specific esterases) and nonspecific esterases (α and β). The esterases can be detected by staining acrylamide gels electrophoresed by samples in presence of α- or β-napthylacetate (for specific esterases) or together (for nonspecific esterases) (Simms, 1965). Occurrence of esterases in numerous isoforms at distinct genetic locus, codominant inheritance and high degree of variability in banding pattern has made esterases a reliable marker for studying genetic variability within and among populations (Eguchi *et al*., 1965).

Intra- and inter-breed genetic variability in silkworms has been reported by analysing polymorphism based on esterase isozymes (Staykova *et al*., 2003; Moorthy *et al*., 2007a, b; Staykova, 2008). Moreover, ontogenetic changes in the esterase profiles of different tissues *viz*. hemolymph, silk gland, fat body, mid gut, genital organ, ovaries and mature eggs of silkworm have also been investigated (Staykova *et al*., 2003). Eguchi *et al*. (1965) conducted genetic and electrophoretic studies on blood esterases of silkworm and described four (Bes^A, Bes^B, Bes^C and Bes^O) fundamental types of blood esterases controlled by codominant alleles and studied their inheritance. Gamo (1978) linked *Bes* (Blood

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Table 1 Morphological characters of silkworm breed

Breeds	Egg colour	Larval marking	Cocoon colour	Cocoon shape	Type
Nistari	Yellow	Marked	Yellow	Spindle with one end pointed	Nondiapausing
Cambodge	Light yellow	Plain	Yellow	Spindle	Nondiapausing
D6(P)N	Light yellow	Marked	White	Dumbbell	Diapausing
SK4C	Yellow	Marked	White	Dumbbell	Diapausing
D6(P)	Light yellow	Plain	White	Dumbbell	Diapausing
SK4	Colour less	Marked	White	Dumbbell	Diapausing

esterase) genes on 11th chromosome with reference to phenotypic markers. Among the blood esterases of *B. mori*, Bes^B, a major esterase has been purified and characterized as carboxylesterase (Arai *et al*., 2000). Despite the use of esterases as markers for genetic diversity studies (Staykova *et al*., 2008) they are also known to participate in thermotolerance mechanism in insects (Moorthy *et al*., 2007c, 2008; Chattopadhyay *et al*., 2001). Positive relationship has also been found between the activity of heat stable esterase of mid gut and thermotolerance in temperate silkworm breeds (Wu and Hou, 1993). Heat stable esterase has also been identified in hemolymph of tropical silkworm breeds (Chattopadhyay *et al*., 2001).

The mulberry silkworm, *Bombyx mori* is an insect with economic importance, domesticated for more than 5000 years within a narrow range of rearing temperature of 25 - 28 °C. Therefore, silkworms are vulnerable to above or below this temperature range. Particularly, rearing silkworms at higher temperatures have adverse effects on their survivability and silk yield (Kumar *et al*., 2012). Thermotolerance in *B. mori* is influenced by genetic and environmental factors (Kumar *et al*., 2012). Moreover, the thermotolerance in *B. mori* is measured in terms of survival rate (Kumar *et al*., 2012), expression of heat shock proteins (Velu *et al*., 2008), activity of catalase enzyme (Nabizadeh and Kumara, 2011) and synthesis of heat stable esterases at high temperature conditions (Moorthy *et al*., 2008). While several studies are linked with detection, profiling and quantifying activity of heat stable esterases in *B. mori* (Chattopadhyay *et al*., 2001; Moorthy *et al*., 2008; Somasundaram *et al*., 2009; Patnaik *et al*., 2012; Neerati *et al*., 2013), far few studies are associated with their classification (Velu *et al*., 2008). Furthermore, based on substrate-inhibitor specificities, esterases are classified as arylesterases, carboxylesterases and cholinesterases (Yoo *et al*., 1996). Therefore, this study is aimed to identify the heat stable α-esterase and classify the hemolymph esterases including the heat stable esterase of *B. mori* through inhibition studies.

Materials and Methods

Silkworm breeds and high temperature treatment

Six silkworm breeds comprising of two multivoltines (Nistari and Cambodge), two bivoltines [D6(P) and SK4] and their near isogenic lines (NILs) [D6(P)N and SK4C] were considered for this study. Pure lines of all the breeds for this study were obtained from Central Sericultural Research and Training Institute (CSRTI), Berhampore, West Bengal, India. D6(P)N and SK4C are near isogenic lines of D6(P) and SK4, respectively. D6(P)N was developed by using D6(P) as recurrent parent and Nistari as donor parent, similarly, SK4C was developed by using SK4 as recurrent parent and Cambodge as donor parent (Moorthy *et al*., 2007b). The details of morphological characters of the selected silkworm breeds are presented in Table 1.

Selected silkworm breeds were reared from hatching to 2nd day of 5th instar at 25 ± 1°C as recommended by Krishnaswami *et al*. (1978). Three replicates with 300 larvae each were reared. On 3rd day of 5th instar, the larvae were exposed to five different temperature regimes *viz*. 25 ± 1, 32 ± 1, 34 ± 1, 36 ± 1 and 38 ± 1 °C in a SERICATRON (chamber for temperature and humidity control) for 6 h a day to till they started spinning. The larvae were fed twice a day with mulberry leaves. Matured larvae were picked and mounted on plastic mountages to spin cocoons. Cocoon harvesting was carried out on the 7th day of spinning and defective ones were removed. The number of larvae that survived high temperature treatment, formed healthy cocoons and able to metamorphose to pupa, such cocoons were considered and counted as survival percentage. Data on economically important quantitative traits *viz*., fecundity, cocoon yield/10,000 larvae by weight (kg), single cocoon weight (g), single shell weight (g) and shell percent (%) were collected at 25 ± 1 °C as described by Krishnaswami *et al*. (1978). Data on survival percentage of selected silkworm breeds at different high temperature regimes was also collected.

Table 2 Quantitative traits of the silkworm breeds reared at 25 ± 1ºC presented as mean ± SD. Means sharing different letters are significantly different

Breeds	Fecundity	Larval weight (g)	Cocoon yield / 10,000 larvae by weight (kg)	Single cocoon weight (g)	Single shell weight (g)	Shell percent
Nistari	312 ± 9.12^{a}	23.28 ± 0.45^{a}	9.89 ± 0.16^{a}	1.18 ± 0.05^{a}	0.168 ± 0.002^{a}	14.25 ± 0.026^{a}
Cambodge	345 ± 14.26^{b}	25.66 ± 0.71^{b}	9.641 ± 0.33^{b}	1.23 ± 0.02^{a}	0.179 ± 0.002^{b}	14.54 ± 0.402^{a}
D6(P)N	485 ± 19.50^{c}	33.57 ± 1.65^{c}	13.587 ± 0.04^{c}	1.53 ± 0.03^{b}	0.298 ± 0.003^{c}	19.36 ± 0.220^{b}
SK4C	512 ± 12.50^{d}	34.42 ± 0.50^{c,d}	14.087 ± 0.18^{c,d}	1.61 ± 0.01^{c}	0.333 ± 0.001^{d}	20.51 ± 0.173^{c}
D6(P)	560 ± 15.50^{e}	36.82 ± 1.94^{c,e}	14.362 ± 0.04^{c,e}	1.62 ± 0.01^{c,d}	0.334 ± 0.003^{d,e}	20.58 ± 0.152^{c,d}
SK4	520 ± 16.20^{d,f}	36.95 ± 0.49^{c,f}	13.820 ± 0.66^{c,f}	1.57 ± 0.05^{b,c,e}	0.307 ± 0.003^{c,f}	19.50 ± 0.195^{b,e}

Hemolymph collection

Hemolymph was collected separately from the larvae of selected silkworm breeds reared at 25 ± 1 °C on the 5th day of 5th instar by cutting a proleg and bleeding into a pre-chilled microfuge tubes with 1 mg of phenylthiourea to avoid the activity of prophenol oxidase leading to melanization of hemolymph. Each sample was pooled from five larvae to minimize variations. The hemolymph was centrifuged at 1500*g* for 10 min at 4 °C and the supernatant was stored at -20 °C until use.

Polyacrylamide gel electrophoresis

Hemolymph samples were electrophoresed on 7.5 % polyacrylamide gels with Tris-glycine electrode buffer of pH 8.3 under non-denatured conditions using dual vertical gel electrophoresis system (Omega) connected with thermo-controlled water bath (Pharmacia LKB - MultiTemp II). The procedure was followed as described by Harris and Hopkinson (1970). The haemolymph samples were electrophoresed under constant voltage of 110 V at 4 °C until the tracking dye reached the bottom of the gel. Following electrophoresis, gels were soaked in a boric acid (0.5 M, pH 5) for 30 min at 4 ºC. Traces of boric acid were removed by washing gels for two times with ice cold distilled water. The gels were stained with α-esterase substrate (2 % or α-napthyl acetate in acetone), 50 mg of fast blue BB salt and 100 ml of 0.1 M phosphate buffer pH 7.0 for 1 - 2 h at room temperature by following the procedure of Simms (1965). Gels were photographed and relative mobility (R_f values) of each esterase band was calculated by using the formula: R_f = Distance of protein migration / Distance of dye migrated. Esterase bands were designated as Est-1, Est-2, Est-3 and so on from the anodal migration to cathode.

Identification of heat stable esterases

For identification of heat stable esterases, the gels were incubated at 60 ± 1 ºC for 15 min in a water bath prior to addition of α-napthyl acetate. Following incubation, the gels were stained for esterase activity by following aforesaid procedure. Esterase bands that were able to retain the activity at higher temperatures were considered as heat stable esterases.

Inhibition of esterases

Phenylmethylsulfonyl fluoride (PMSF) and eserine sulphate were used as inhibitors in this study. For inhibition analysis, gels were incubated in 50 ml of phosphate buffer (0.1 M, pH 7.0) containing 200 µl of inhibitor (final concentration of 0.2 mM) for 1 h at room temperature. Following incubation, the gels were stained for esterase activity by following aforesaid procedure in presence of inhibitor. Esterase bands that were able to retain the activity were considered to be unaffected by the inhibitors while the esterase bands which were unable to retain their activity were considered to be affected by the inhibitors.

Statistical analysis

Data on fecundity, cocoon yield/10,000 larvae by weight, single cocoon weight, single shell weight, shell percent and survival percentage of selected silkworm breeds at 25 ± 1 °C were collected. Additionally, survival percentages of the selected silkworm breeds at different high temperature regimes were also collected. The collected data on

Table 3 One-way ANOVA on each quantitative trait of silkworm

Quantitative traits	*DF*	Mean square	*P* value
Fecundity	5	30900.089	0.0001
Larval Weight	5	0.131	0.0001
Cocoon yield / 10,000 larvae by weight	5	9.762	0.0001
Single cocoon weight	5	0.121	0.0001
Single shell weight	5	0.319	0.0001
Shell percent	5	25.815	0.0001
Survival percentage at 25°C	5	21.173	0.0001
Survival percentage at 32°C	5	12821307.59	0.0001
Survival percentage at 34°C	5	870.309	0.0001
Survival percentage at 36°C	5	1283.602	0.0001
Survival percentage at 38°C	5	28.387	0.000 I

different quantitative traits were subjected to one way analysis of variance (ANOVA) with silkworm breeds as factor. Prior to analysis the data was tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test) otherwise, after appropriate transformations to meet the criteria of parametric analysis. Tukey HSD *post hoc* test was conducted for detecting significant differences ($p < 0.05$) between means. All statistical analyses were performed using SPSS 11.5 statistical package.

Results

Variations in the quantitative traits at 25 ± 1 °C

Generally, multivoltine breeds had low values than bivoltine breeds for all the quantitative traits studied. NILs had intermediate values compared to their parents that is of higher than their multivoltine parents and lower than bivoltine parents (Table 2). Moreover, the performance of NILs was almost equal to their bivoltine parents than multivoltine parent. Fecundity was highest and lowest in D6(P) (560 ± 15.50) and Nistari (312 ± 9.12), respectively. Highest and lowest larval weight was noted in SK4 (36.95 ± 0.49 g) and Nistari (23.28 ± 0.45 g), respectively. Highest and lowest cocoon yield/10,000 larvae by weight was noted in D6(P) (14.362 ± 0.04 kg) and Nistari (9.641 ± 0.33 kg), respectively. D6(P) (1.62 ± 0.01 g) and Nistari (1.18 ± 0.01 g) displayed highest and lowest single cocoon weight. Single shell weight and shell percent were highest and lowest in D6(P) (0.334 ± 0.003 g, 20.58 ± 0.152 %) and Nistari (0.168 ± 0.002 g, 14.25 ± 0.026 %), respectively. One-way ANOVA revealed significant differences ($p < 0.05$) in all the quantitative traits studied (Table 3).

Survival percentage of selected breeds at high temperatures

In order to understand level of thermotolerance in selected silkworm breeds, they were exposed to different high temperatures *viz*. 32 ± 1, 34 ± 1, 36 ± 1 and 38 ± 1 °C and survival percentages of all breeds were collected. The larvae reared at 25 ± 1 °C were considered as normal and had better survival. Survival percentage was decreased as the rearing temperature was increased in all the breeds. Multivoltine breeds (Nistari and Cambodge) exhibited higher survival followed by NILs [D6(P)N, SK4C] and bivoltine breeds [SK4 and D6(P)]. Of the multivoltine breeds, Nistari displayed highest survival percentages at each tested temperatures with overall mean of 82.91 %. Similarly, among NILs and bivoltine breeds, SK4C and SK4 displayed highest survival percentages at each tested temperatures with overall mean of 70.03 % and 48.57 % respectively. The overall means of survival percentage of Cambodge, D6(P)N and D6(P) were 79.34 %, 65.99 % and 41.80 % respectively. Interestingly, NILs had survival percentages higher than their bivoltine parents and lower than their multivoltine parents at high temperatures. Significant differences ($p < 0.05$) were observed between survival percentages of NILs and their parents at each tested temperatures (Fig. 1).

Esterase isozyme polymorphism

Five different isoforms of α-esterases were observed in this study. Starting from the anode end the esterase isoforms were designated as Est-1 (R_f -0.425), Est-2 (R_f -0.346), Est-3 (R_f -0.295), Est-4 (R_f -0.247) and Est-5 (R_f -0.217). Of the five esterases, two were monomorphic (Est-1 and Est-2) and three were polymorphic (Est-3, Est-4 and Est-5) between

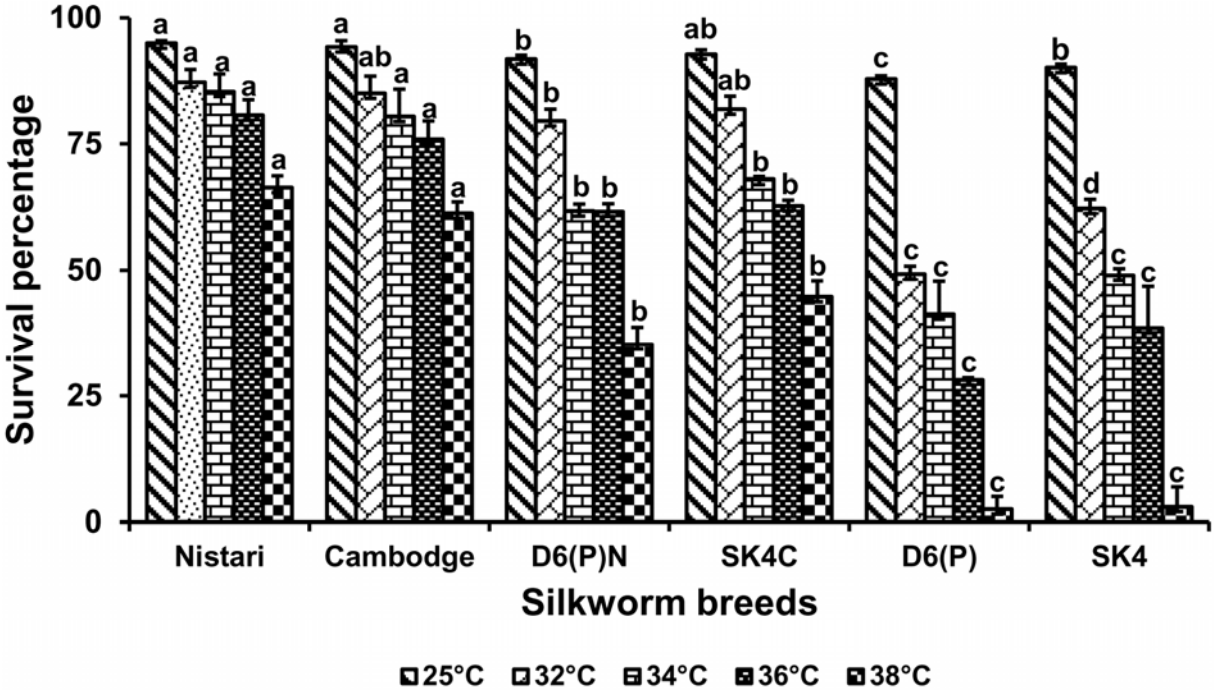

Fig. 1 Survival percentages of different silkworm breeds reared at different high temperature regimes. Data represented in mean ± SD. Means sharing different letters are significantly different.

the selected silkworm breeds accounting for 60 % polymorphism. Est-1 and Est-2 were expressed in all the selected breeds. Est-3 was expressed in NILs [D6(P)N and SK4C] and multivoltine parents (Nistari and Cambodge) but was not expressed in the bivoltine parents. Est-4 was expressed only in D6(P). Est-5 was expressed in NIL, SK4C and its bivoltine parent, SK4. The highest number of esterases was expressed (four) in SK4C, while three esterases were expressed in each of the other breeds. The esterase isozyme banding pattern of NILs (D6(P)N and SK4C) was very similar to their multivoltine parents (Nistari and Cambodge) with an exception of Est-5 in SK4C, which was inherited from its bivoltine parent SK4 (Fig. 2).

Identification of heat stable esterases

Acrylamide gels electrophoresed with hemolymph samples were incubated at 60 ± 1 ºC for 15 min in a water bath following staining with α-napthyl acetate as said before. While the heat liable esterases disappeared, heat stable esterases retained their activity. In this study, Est-1, Est-4 and Est-5 were found to be heat liable and, Est-2 and Est-3 were identified as heat stable (Fig. 3).

Classification of esterases

Inhibition studies using PMSF and eserine sulphate as substrate inhibitors were carried out to classify the hemolymph α-esterases. The inhibition studies on hemolymph α-esterases showed that Est-1 and Est-5 were totally inhibited by eserine sulphate and PMSF respectively, while Est-3 was totally inhibited by both inhibitors. But Est-2 and Est-4 were unaffected by both PMSF and eserine sulphate (Figs 4A, B).

Discussion

Esterases represent a large, diverse and multifunctional group of hydrolytic enzyme systems that possess the property of overlapping substrate specificity, hydrolysing both endogeneous and exogenous esters of widely differing structures leading to hindrance of identification and classification (Dixon and Webb, 1979; Walker and Mackness, 1983). In silkworm, most of esterase isozyme polymorphism studies have done with bivoltine breeds and very little work has been conducted on multivoltine breeds (Chattopadhyay *et al*., 2001). Our study concentrated on multivoltines, bivoltines and their NILs. This study revealed expression of five esterases, of which three were polymorphic and two were breed specific. Esterase with R_f -0.247 (Est-4) was specific to D6(P) and another esterase with R_f -0.295 (Est-3) was expressed only in NILs and multivoltine breeds. Another esterase with R_f -0.217 (Est-5) was expressed in SK4 and its near isogenic line SK4C. Est-3 was specific to multivoltine silkworm breeds and same was inherited by their NILs. While developing NILs, Est-3 was used as marker to track the multivoltine blood characterized with higher survival. Therefore, NILs had higher survival than their bivoltine parents (Moorthy *et al*., 2007a).

Heat stable esterases have been reported in plants (Pandey, 1973), fishes (Okumura *et al*., 1981) and in model insects such as *Drosophila*

(Cochrane, 1976). Wu and Hou (1993) also observed one heat stable α-esterase band (*i.e.*, Est-5) which tolerated 60 °C and was found abundant in the mid gut tissue of temperate silkworm breed. Chattopadhyay *et al*. (2001) observed a heat stable β-esterase and an Est-3 in the haemolymph of tropical silkworm breed. In our earlier study (Moorthy *et al*., 2007c), we reported the differential expression of esterases (Est-1 and Est-3) in the mid gut and fat body tissues of Nistari breed at different temperature regimes ranging from 0 ºC to 32 ºC. However, the differences in the esterases of fat body tissue were not significant, while in the mid gut the expression of Est-1 was high at 32 ºC followed by 0 ºC, 25 ºC and 15 ºC. In case of Est-3, the expression was comparatively higher at 0 ºC, than 32 ºC and 15 ºC. Recently, Patnaik *et al*. (2012) reported a non-specific esterase (Est-3) and a specific α-esterase (Est-3) as heat stable esterases in the haemolymph of *B. mori*.

In this experiment, two esterases *viz*. Est-2 and Est-3 were found to be heat stable while Est-1, Est-4 and Est-5 were heat liable. Though, Est-2 was heat stable, it was monomorphic and was expressed in all the studied silkworms. Therefore, it had little effect on the specific thermotolerance with respect to breeds. Contrary to it, Est-3 was expressed in multivoltines and NILs but not in bivoltines, which were sensitive to high temperatures. Thus, it can be opined that the heat stable Est-3 inherited from multivoltine parents to the developed NILs followed directional selection (selection based on Est-3) during the course of breeding. Generally, characters under stringent selection tend to be conserved and inherited in populations, while those under less stringent selection vary highly. This mode of selection was made conserved and heritable in the successive generations by strictly selecting larvae that are tolerant to 33 ºC with the esterase banding pattern similar to multivoltine parents for breeding successive generations.

According to Staykova *et al*. (1998) 'Biochemical marker' is a term used for biochemical macromolecules, which are able to differentiate between two species or different biotypes of the same species. Such biochemical markers are also used to identify the breeds resistant to pesticides. Hemolymph is a rich source of biochemical compounds in *B. mori*, of which enzymes are less changeable with respect to their genetic structure compare to other biochemical compounds, proving that they are good biochemical markers. Further, better breeding program in the silkworm can be designed by identification of biodiversity (Etebari *et al*., 2005). In this study, we have identified one (Est-4) breed specific esterase, two esterases inherited to NILs, one each from bivoltine (Est-5) and multivoltine parent (Est-3). This result reveals that the esterases identified can differentiate between the silkworm breeds under study, more clearly between bivoltine and multivoltine silkworm breeds. Est-3, inherited from multivoltine parents to their respective NILs was heat stable and was the only marker tracking the multivoltine blood in each generations of NIL development. Presence of breed specific esterases in D6(P) (Est-4) and SK4 (Est-5)

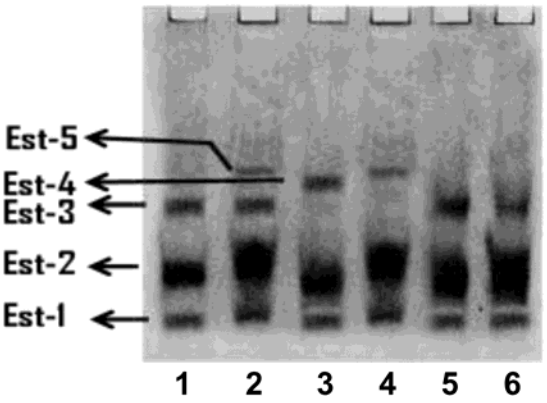

Fig. 2 Esterase isozyme pattern in the haemolymph on 5th day of 5th instar larvae of silkworm breeds reared at 25 ± 1°C. Lane 1 and 2 are NILs [D6(P)N and SK4C], 3 and 4 are bivoltine parents [D6(P) and SK4] and 5 and 6 are multivoltine parents (Nistari and Cambodge).

and absence of (Est-3) did not increase survivability in bivoltines, but their respective NILs had increased survivability at high temperatures, which had heat stable esterase (Est-3) inherited from their multivoltine parents. Therefore, Est-3 is a prominent esterase that was able to differentiate the thermotolerant and thermo susceptible breeds under study. Hence, Est-3 can be employed as a biochemical marker to classify the silkworm breeds based on their thermotolerance ability inherited from their multivoltine parents. Est-3 is characteristic feature of thermotolerant multivoltine breeds integrated in NILs. Therefore, selection of parents based on such biochemical markers could be an effective approach for successfully breeding high temperature tolerant silkworm breed. The presence of two breed specific and one multivoltine specific esterases observed in this study supports that esterases can be used as reliable marker for breed identification. Furthermore, screening of the entire silkworm germplasm for the esterases would generate valuable information that can serve to identify the silkworm breeds.

Patnaik *et al*. (2012) suggested that characterization of candidate heat stable esterases will enlighten its suitability as markers towards heat resistance and could be explored in the future breeding programs. In this direction, the characteristics of each esterase isozyme can be determined by the addition of specific inhibitors during the process of enzymatic staining of gels. Based on substrate-inhibitor specificity, esterases are classified as arylesterases, carboxylesterases and cholinesterases (Yoo *et al*., 1996). In this study, the inhibitors, PMSF and eserine sulphate were used to classify esterases. Inhibition studies revealed that PMSF totally inhibited Est-5 activity but it was unaffected by eserine sulphate. PMSF inhibits carboxylesterases. The PMSF is a serine-hydrolase inhibitor suggests that inhibited esterases contain serine residues in their active sites. Thus, these observations suggest that the Est-5 might be

carboxylesterase. Eserine sulphate totally inhibited Est-1 indicating that it might be cholinesterase. Cholinesterases are important regulatory enzymes that are responsible for controlling the neural transmission on synapses by hydrolyzing acetylcholine, the excitatory of neurotransmitter (Yu *et al*., 2009). Also cholinesterases are targeting on organo-phosphorus and carbamate insecticides, as these toxic compounds readily inhibit those (Baffi *et al*., 2005). Carboxylesterases also serve a protective role for the target cholinesterases during organophosphate intoxication because the carboxylesterases are alternative phosphorylation sites (Watson and Chambers, 1996). Carboxylesterases are also associated with physiological processes of the cuticular wall synthesis and regulation of juvenile hormone levels (Sparks *et al*., 1979). The inhibition pattern for Est-2, Est-3 and Est-4 were complex. Est-3 (heat stable esterase) was inhibited by both PMSF and eserine sulphate which putatively indicates that it represents cholinesterase activity and has serine in its active site. Cholinesterases also have a proteolytic activity in addition to their cholinergic activity, acting as proteases in regulating cell growth and development (Small, 1990). Therefore, cholinesterases (Est-3) might be responsible for higher survival of NILs and their multivoltine parents than bivoltines at high temperatures as Est-3 was not expressed in bivoltines. Est-2 and Est-4 were unaffected by both the inhibitors, therefore it is assumed that they may belong to arylesterases group, but empirical studies are needed to prove this assumption.

Arai *et al*. (2000) characterized an esterase (BesB) in the hemolymph as carboxylesterase using substrate-inhibition studies and enzyme kinetics methods. Yu *et al*. (2009) identified 76 putative carboxylesterases on newly assembled *B. mori* genome through *in silico* analysis. Murthy *et al*. (1996) purified and characterized a carboxylesterase from the mid gut of *B. mori* by inhibition studies in conjunction with other molecular techniques. Recently, Neerati *et al*. (2013) classified the esterases from silk gland of *B. mori* as carboxylesterases by inhibition studies. Qualitative (Chattopadhyay *et al*., 2001; Moorthy *et al*., 2008; Somasundaram *et al*., 2009) and quantitative analysis (Velu *et al*., 2008; Patnaik *et al*., 2012) of heat stable esterases has also been carried out by many researchers in *B. mori*. Accepting the calls from Patnaik *et al*. (2012) to characterise the heat stable esterase, in this study, in addition to the identification of heat stable esterase (Est-3) we have also found through inhibition studies that it represents cholinesterase activity with serine in its active site.

Hemolymph is a liquid open circulatory system with tissues like mid gut, fat body and silk gland embedded in it. Consequently, several proteins including nonsecretory proteins synthesized in the fat body are carried in the hemolymph to specific subcellular sites. These factors are important in the acquisition of thermotolerance (Kampinga, 1993). In this regard, the presence of heat stable esterases in the hemolymph of *B. mori* can be considered as a desirable feature in conferring thermotolerance to the larvae. Therefore, hemolymph was selected as suitable tissue for detecting heat stable esterases.

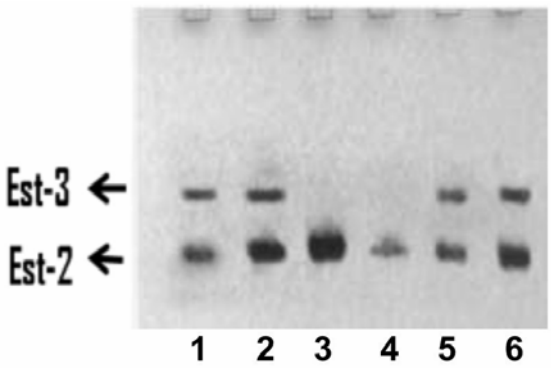

Fig. 3 Heat stable esterases in haemolymph on 5th day of 5th instar larvae of silkworm breeds. Lane 1 and 2 are NILs [D6(P)N and SK4C], 3 and 4 are bivoltine parents [D6(P) and SK4] and 5 and 6 are multivoltine parents (Nistari and Cambodge).

This study also delineates the relationship between multivoltine and bivoltine breeds, and their NILs with reference to quantitative traits. Except survivability, bivoltine breeds had higher values for all quantitative traits than the multivoltine breeds. Since, bivoltines are originated from temperate regions and multivoltines from tropical regions, they have different genetic backgrounds. Consequently, the bivoltines produce high quality and quantity of silk but sensitive to high temperatures, whereas, multivoltines can tolerate high temperatures but produce low quality and quantity of silk (Moorthy *et al*., 2007a; Kumar *et al*., 2012). But NILs, SK4C and D6(P)N showed intermediate values nearer to their bivoltine parents, SK4 and D6(P) (Table 2). These NILs were developed by backcrossing females of bivoltine parents with F_1 males (♀ bivoltine × ♂ multivoltine) for six generations followed by two generations of self-crossing. In each generation, larvae were screened at 33 °C and esterase banding pattern similar to multivoltine parents (Moorthy *et al*., 2007b). Therefore the variability in quantitative traits can be explained by genetic differences in the breeds because the quantitative traits discussed are mainly controlled by genetic factors (Ahsan *et al*., 2010; Singh *et al*., 2011). It is clear that the studied traits are quantitative in nature and are controlled by many quantitative trait loci (QTLs) present on multiple chromosomes. Most probably, during the development of NILs most of the QTLs for the studied traits are inherited from the recurrent bivoltine parents by their respective NILs. Thus, the NILs performed almost equally to their respective bivoltine parents. Exception to this result is survival percentage which is directly associated with thermotolerance trait. In case of survival percentage, NILs had higher survival percentages than their bivoltine parents and lower than their multivoltine parents because thermotolerance is also controlled by many quantitative trait loci (QTL)

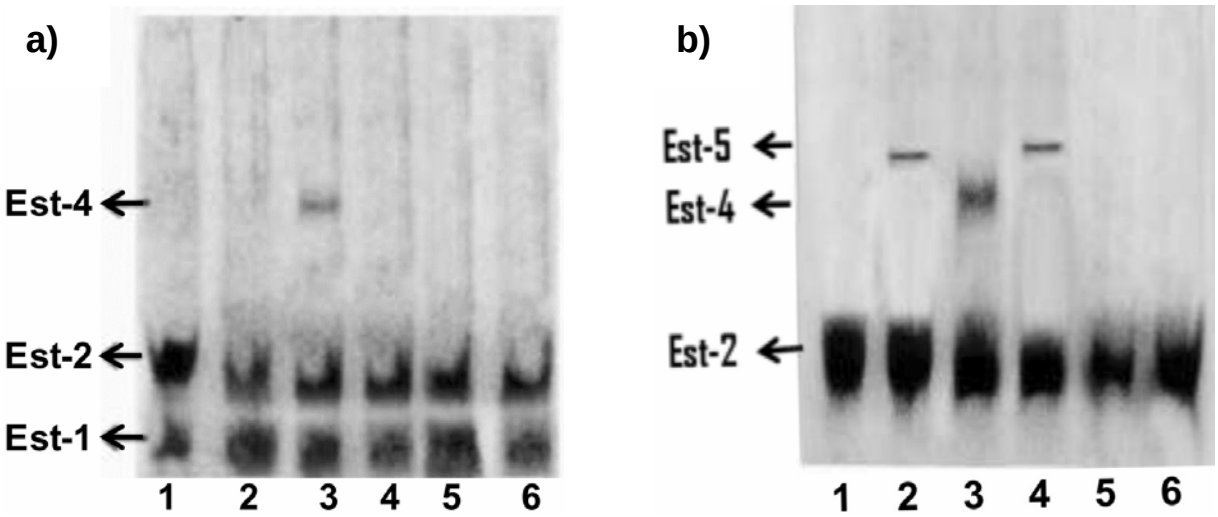

Fig. 4 a) Inhibition of esterases by PMSF in haemolymph on 5th day of 5th instar larvae of silkworm breeds. Lane 1 and 2 are NILs [D6(P)N and SK4C], 3 and 4 are bivoltine parents [D6(P) and SK4] and 5 and 6 are multivoltine parents (Nistari and Cambodge). b) Inhibition of esterases by eserine sulphate in haemolymph on 5th day of 5th instar larvae of silkworm breeds. Lane 1 and 2 are NILs [D6(P)N and SK4C], 3 and 4 are bivoltine parents [D6(P) and SK4] and 5 and 6 are multivoltine parents (Nistari and Cambodge).

present on multiple chromosomes. It is obvious that all the QTLs for thermotolerance are not inherited by NILs from their multivoltine parents. Because the multivoltine parents are involved in a single cross that is in the development of F_1 hybrid resulting in partial inheritance of QTLs linked to thermotolerance from their multivoltine parents. This partial number of QTLs (but not all) were activated and retained in the successive generations by rearing and exposing the larvae to high temperature of 33 °C and selecting the tolerant larvae for successive generations. Hence, NILs had intermediate values for survival percentages compared to their parents. Therefore, esterase polymorphism at distinct genetic loci might also be linked to the changes observed in the quantitative traits between NILs and their parents.

The heat stable Est-3 which is present in the NILs and their multivoltine parents might be responsible for their higher survival percentages than the bivoltines at tested high temperatures. Nevertheless, the introgression of Est-3 in the NILs during breeding might be one of the major factors that led to the increase in its thermotolerance capacity than their bivoltine parents. The overall means of survival percentage at all the tested temperatures of D6(P)N and SK4C (NILs) were 1.58 and 1.44 times more than D6(P) and SK4 (Bivoltine parents), respectively and 0.8 and 0.88 times lower than Nistari and Cambodge (Multivoltine parents), respectively. The integration of Est-3 in the NILs during breeding coupled with heat selection indicates the retained thermotolerance character from the multivoltine breed in each generation because expression of Est-3 increased survivability in NILs as well as in multivoltines but expression of other esterases did not increased or decreased the survivability in bivoltine breeds. This selection phenomenon was adopted from Wu and Hou (1993), they also proved that heat stable esterase activity was increased with thermotolerance by observing the heat stable esterase activity for 7 generations. In *Drosophila* species also, the survival was increased from 35 % to 64 % after selecting 10 generations at 40 °C for 30 min. Recently, heat stable Est-1 was successfully integrated in the NILs of CSR2, a productive bivoltine breed. This marks the introgression of multivoltine thermostable factors into the bivoltine silkworm breed 'CSR2' leading to improved survivability measured in terms of pupation percentage (Das *et al*., 2013). Though thermotolerance in *B. mori* is controlled by multiple genetic factors, this study shows that expression of heat stable esterases is one of the major factors among them. Furthermore, complete transcriptome analysis in multivoltines and bivoltines with their NILs at high temperature conditions is required to understand the molecular mechanism involved in thermotolerance in *B.mori*.

This study confirms that the NILs [D6(P)N and SK4C] have inherited most of the QTLs linked to silk quality and quantity from their bivoltine parents [D6(P) and SK4] and that of thermotolerance from their multivoltine parents (Nistari and Cambodge). Similarly, Chatterjee *et al*. (1993) and Chatterjee and Datta (1992) reported that digestive juice amylase had a positive correlation with survivability and a negative correlation with larval weight, larval duration, single cocoon weight and single cocoon shell weight. Alkaline phosphatase and invertase also had positive roles in the expression of yield attributes. However, further investigation is needed to correlate the variations in the quantitative traits with esterase activity.

Results of this study indicate that esterase can be used as a reliable biochemical marker for diversity studies and breeding programs associated with thermotolerance in *B. mori*. This work also describes the classification of heat stable hemolymph esterase (Est-3) through inhibition studies in *B. mori*. The information generated from this study would be useful for understanding of molecular and functional mechanisms involved in esterase-mediated thermotolerance in *B. mori*.

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